INDUCTION OF METAMORPHOSIS BY ECDYSONE ANALOGUES : *DROSOPHILA* IMAGINAL DISCS CULTURED *IN VIVO*

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The imaginal discs of *Drosophila* provide an increasingly attractive system for the study of development (see *e.g.*, Gehring, 1968). One such developmental problem is the hormonal control of metamorphosis. This problem and others could be investigated more easily if metamorphosis could be induced *in vitro*. The early literature on the somewhat disappointing attempts at *in vitro* culture of insect tissues has been reviewed by Day and Grace (1959). Imaginal discs cultured *in vitro* without ring glands do not metamorphose, whereas, when ring glands are added to the medium, the discs will metamorphose (Kuroda and Yamaguchi, 1956; Gottschewski, 1960; Schneider, 1964, 1966). Experiments with crystalline molting hormone, ecdysone, have been less successful. Oberlander and Fulco (1967) and Oberlander (1969) have reported that the only observable effects of ecdysone, ecdysterone, or inokosterone on wing discs of the moth *Galleria mellonella* cultured *in vitro* were tracheal growth and occasional elongation. Burdette, Hanley and Grosch (1968) cultured eye-antennal discs of *Drosophila in vitro* and in adult abdomens with the addition of ecdysone extracts from *Bombyx mori*. Although ecdysones accelerated pigment deposition and thickening of the discs, no bristles were found, and cuticle formation was not mentioned. Thus, in experiments reported to date, ring glands cause metamorphosis of imaginal discs *in vitro*, but crystalline molting hormone has not yet been found to have this effect. Bodenstein (1943) and Vogt (1944) have shown that *Drosophila* imaginal discs cultured in the abdomen of the adult fail to metamorphose, unless larval ring glands are also transplanted into the host. Since ring glands will cause metamorphosis of imaginal discs both *in vitro* and *in vivo*, whereas crystalline molting hormone has not been shown to cause metamorphosis *in vitro*, it seemed reasonable to test the effects of crystalline molting hormone on imaginal discs cultured *in vivo* before proceeding with further tests *in vitro*. The results to be reported here show that ecdysterone can stimulate complete metamorphosis of *Drosophila* imaginal discs cultured in the adult abdomen. Preliminary results have been reported previously (Postlethwait and Schneiderman, 1968).

MATERIALS AND METHODS

Imaginal first leg discs of mature third instar male and female larvae of an Oregon R stock of *Drosophila melanogaster* were dissected in Ephrussi-Beadle Ringer solution. For most experiments, one of the paired first leg discs from each

larva was used as the control, the other was treated with hormone. Each whole disc was transplanted by the Ephrussi and Beadle (1936) technique (see also Ursprung, 1967) into the abdomen of a three-day-old fertilized female adult fly of the same stock. A large needle, about 150 μ in diameter, was employed to reduce damage to the discs, since cut or damaged discs proliferate more than uninjured imaginal discs (Tobler, 1966). The hosts were allowed to recover from the implantation for twenty-four hours, and then were injected with 0.2 μl of either hormone or control solution. In some experiments the injections were repeated on succeeding days. Although only four days are required to complete metamorphosis *in situ*, Bodenstein (1943) found that leg discs required at least nine days to metamorphose when they were cultured in adult hosts in which ring glands had been implanted. We chose to wait eleven days before retrieving the discs in our experiments.

Solutions of ecdysterone (20-hydroxyecdysone, iso-inokosterone) obtained from Rohto Pharmaceutical Company, Osaka, Japan, or cyasterone, generously supplied by Professor Carroll Williams, were made up in 10% ethanol in Ringer. Control animals received 10% ethanol in Ringer without hormone. The mortality rates for control and experimental animals were low and approximately equal. More than 170 hormone-treated implants and 70 control discs were recovered.

The imaginal leg disc consists of a columnar epithelium covered by the peripodial membrane, forming a cellular sac (Auerbach, 1936). The surface of the cells which secretes the cuticle faces the lumen of the sac. During normal pupation, the disc everts: the columnar epithelium enlarges by changes in cell shape (Poodry and Schneiderman, in preparation), and pushes through the peripodial sac so that the chitogenous surface comes to lie on the outside of the anlage. A thin, transparent pupal cuticle is secreted, followed by a melanized, bristle-bearing adult cuticle. These phenomena were examined in the experimental material. Eversion was observed directly in freshly-dissected experimental discs. However, in some experiments, eversion was obscured by subsequent changes. In these cases, a convenient and reliable method of determining relative volumes of imaginal discs was to measure the largest dimension of an unfixed disc in three mutually perpendicular planes with an optical micrometer, and then compute the volume. To allow comparison with the *in situ* situation, the data presented in the tables give the size of the discs relative to the size of the leg disc from the mature third instar larva. The pupal and adult cuticles were observed in Gomori-stained whole mounts (Melander and Wingstrand, 1953), and in sections stained with Mallory's triple stain.

RESULTS

Series I: Single and multiple doses of ecdysterone

Preliminary experiments employed single doses of ecdysterone at final concentrations of 0.01 to 100 μg/g host. The results revealed only a slight stimulation of disc enlargement and no cuticle secretion. Since Bodenstein (1943) found that transplanted ring glands continuously secrete hormone, since the prothoracic gland is necessary for several days for metamorphosis of silk worm pupae (Williams, 1952), and since ecdysterone is readily inactivated by some insects (Ohtaki, Milkman, and Williams, 1968), it seemed likely that a sustained supply of ecdysterone

might be necessary for metamorphosis. Therefore in Series I, ecdysterone was injected in multiple as well as single doses into adult abdomens containing discs. The total amount of ecdysterone injected in both cases was equal. The discs were transplanted into female hosts on day one. About twenty-four hours later, all of the experimental and control animals were injected with ecdysterone. One experimental group received a total dose of 7.2 $\mu g/g$, and a second group, a total dose of 720 $\mu g/g$. The results are shown in Table 1. Although the total amount delivered in single- and multiple-dose experiments was the same, discs receiving the total dose in instalments enlarged more and secreted cuticle more often than did
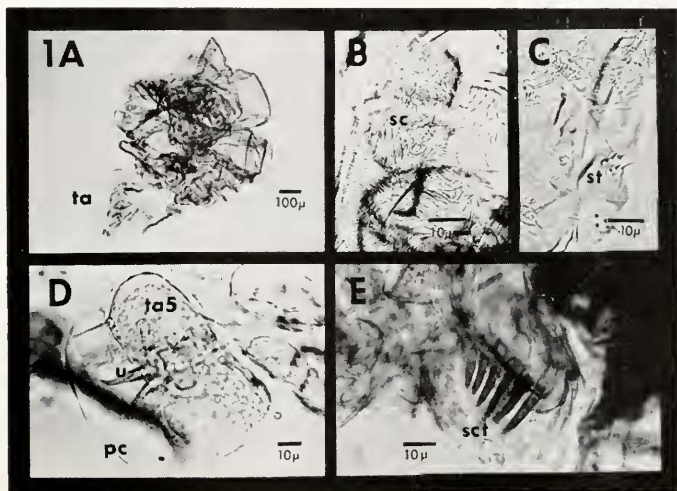

FIGURE 1. Adult structures produced by imaginal leg discs induced to metamorphose by ecdysterone; (A) A metamorphosed leg, note everted tarsus; (B) Sensilla campaniformia of the femur; (C) Sensilla trichodea of the coxa; (D) Claw organ, note pupal cuticle enveloping the tarsus; (E) Sexcomb teeth, typical of the male basitarsus. Abbreviations: pc, pupal cuticle; sc, sensilla campaniformia; sct, sex comb teeth; st, sensilla trichodea; ta, tarsus; ta5, fifth tarsal segment; u, unguis.

discs receiving the same amount of hormone in one dose. Apparently, ecdysterone is necessary as a sustained stimulus for metamorphosis, and not merely as a trigger for development. No bristles, hairs or sensillae were formed in Series I.

Series II: Cyasterone

Cyasterone is an analogue of ecdysone extracted from plants which appears to be more potent than ecdysterone in certain systems (Williams, 1968). Table II shows that a single dose of cyasterone stimulated a greater increase in size and a higher frequency of cuticle secretion than an equivalent dose of ecdysterone. The discs treated with one 720 $\mu g/g$ dose of cyasterone were larger than discs treated with one 720 $\mu g/g$ dose of ecdysterone, but were about equal in size to those receiving six injections of 120 $\mu g/g$ ecdysterone. Whole mounts and sections of these implants showed that a single dose of cyasterone was also more effective in promoting cuticle secretion than a single dose of ecdysterone. Cyasterone appears to be either more stable or intrinsically more potent than ecdysterone.

TABLE I

Series I. Single and multiple doses of ecdysterone

	Mature discs	0 µg/g Control	Total dose of			
			7.2 µg/g		720 µg/g	
			One injection of 7.2 µg/g	Six injections of 1.2 µg/g	One injection of 720 µg/g	Six injections of 120 µg/g
Number of discs	98	63	17	24	16	15
Relative size	1.0	1.2	1.2*	2.4	2.0	2.9
% with cuticle	0	3	12	33	6	60

* Not significantly different from controls, $P = 0.05$.

TABLE II

Series II. Single injections of cyasterone and ecdysterone

	Total dose of			
	1.2 µg/g		720 µg/g	
	Ecdysterone	Cyasterone	Ecdysterone	Cyasterone
Number of discs	6	5	16	8
Relative size	1.0*	2.0	2.0	2.9
% with cuticle	0	20	6	25

* Not significantly different from controls, $p = 0.05$.

Series III: Massive doses of ecdysterone

The experiments reported above demonstrate that repeated doses of ecdysterone, or a single large dose of cyasterone induce partial metamorphosis. Since ecdysterone was readily available, we decided to employ massive doses of ecdysterone (1200 µg/g) delivered once, twice, or three times over the course of twelve days. The results are shown in Table III. Although the final size of these discs was smaller than those in Series I, which had received 720 µg/g ecdysterone in six equal installments, or Series II, which had received one 720 µg/g dose of cyasterone,

TABLE III

Series III. Single and multiple injections of large doses of ecdysterone

	Control	Number of treatments with 1200 µg/g ecdysterone		
		1	2	3
Number of discs	63	4	11	18
Relative size	1.2	2.0	2.5	2.4
% with cuticle	3	100	100	100
% with bristles	0	50	100	100

the discs underwent complete metamorphosis, producing a pupal cuticle, followed by an adult cuticle. All of the normal imaginal structures were formed, including claws, sex combs, sensilla trichodea, sensilla campaniformia, and tibial and tarsal transverse bristle rows (Fig. 1). These appeared to be only two differences between the artificially-metamorphosed adult structures and the corresponding structures *in situ*. First, pigmentation of bristles and cuticle in the experimental situation was irregular, though extensive. Occasionally, bristles in addition to those of the tarsal transverse rows (very pale *in situ*) were unpigmented. Second, the number of bristles formed was often less than in normal imaginal structures. For example, most experimental preparations had only five or six sex comb teeth, whereas the normal male has ten to twelve (Hannah-Alava and Stern, 1957). In one case transdetermination was observed, and both leg and wing structures were found.

Series IV: Eversion

When an imaginal disc is transplanted into a larva, and metamorphoses with the host, it usually fails to evert, but forms a hollow ball with bristles and cuticle secreted on the inside. However, the artificially metamorphosed implants in Series III everted, as judged by the exterior orientation of the bristles. This process

TABLE IV

Eversion induced by one dose of 1200 μg g ecdysterone

	Mature discs	Control	1200 μg g ecdysterone
Number of discs not everted	22	12	1
Number of discs everted	0	0	6
Relative size	1.0	1.2	2.6

was examined in Series IV. Leg discs were injected into adult females, and twenty-four hours later the hosts received a single injection of ecdysterone (either 120 μg/g or 1200 μg/g). Twenty-four hours later, the discs were retrieved and examined. The results are recorded in Table IV, and in Figure 2. Ecdysterone clearly stimulated leg disc eversion, and larger amounts of hormone gave greater stimulation. Table IV shows that in the first twenty-four hours after hormone injection, the discs enlarged greatly. When compared to discs cultured for longer periods (Series I and II), it is seen that the disc enlarges most in the first day. Thus, most of the increase in size observed in Series I and II is probably a result of disc eversion.

DISCUSSION

The results show that ecdysone analogues can induce complete metamorphosis of *Drosophila* leg discs cultured in the abdomen of adult female flies. A single dose of hormone was not as effective in causing metamorphosis as the same amount delivered in installments. Ohtaki *et al.* (1968) have shown that α-ecdysone is inactivated rapidly in mature larvae of *Sarcophaga,* and Karlson and Bode (1969)
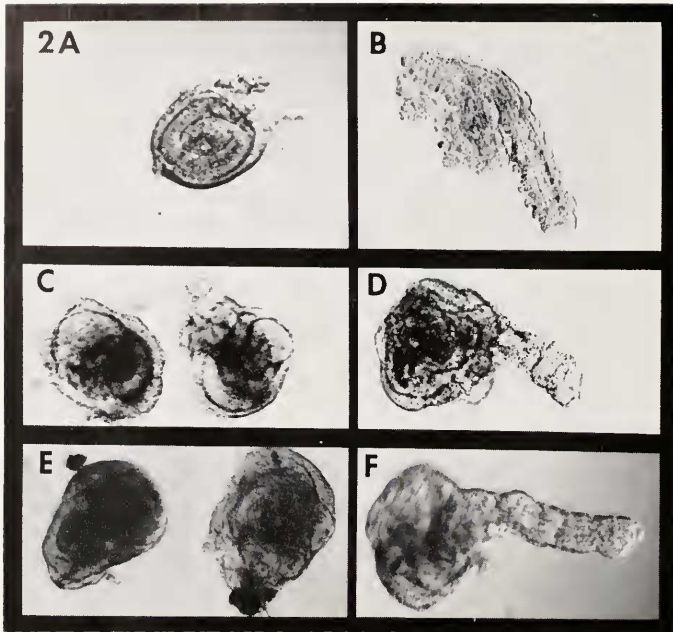
FIGURE 2. Eversion of the imaginal leg disc induced by ecdysterone; (A) Ninety-six hr. imaginal leg disc; (B) Five-hr pupal disc, note eversion; (C) Control discs, 24 hrs after treatment with control solution; (D) Leg disc 24 hrs after treatment with 120 μg/g ecdysterone; (E) Control disc 24 hrs after treatment with control solution; (F) Leg disc 24 hrs after treatment with 1200 μg/g ecdysterone (photograph (B) was taken by C. A. Poodry).

have found that in *Calliphora* its inactivation is due to an enzyme system present in the fat body.   Since ecdysterone is probably also degraded in adult *Drosophila*, and repeated small doses are more effective in inducing metamorphosis than single large doses, we conclude that ecdysterone is necessary as a sustained stimulus, and not merely as a trigger to metamorphosis.

In silkmoths, a high dose (10 μg/g) of ecdysterone causes pupal-adult intermediates (Kobayashi, Takemoto, Ogawa and Nishimoto, 1967; Williams, 1968; Madhavan and Schneiderman, personal communication).   Diptera, however, such as *Musca* larvae treated with a high dose of ecdysterone (300 μg/g) (Kobayashi, et al., 1967) and *Drosophila* imaginal discs treated with a very high dose of ecdysterone (3600 μg/g) show no such abnormalities.   This may be due to rapid inactivation of ecdysterone in Diptera (Ohtaki et al., 1968), or a difference in threshold response to the hormone.

The adult corpus allatum of flies continues to secrete juvenile hormone (Wigglesworth, 1954), which probably is the same factor as the yolk-forming hormone (Wigglesworth, 1948).   Traditional explanations of hormonal control of insect development (Schneiderman and Gilbert, 1964) would predict that a given low level of juvenile hormone might permit deposition of pupal cuticle, and the absence of juvenile hormone would allow adult cuticle to be formed.   The present experiments show, however, that the presence of juvenile hormone in the adult

female host can allow successive production of pupal cuticle and adult cuticle. Perhaps the juvenile hormone titer is too low to exert an effect, or the disc cells have already been determined to secret a pupal cuticle followed by adult structures. Although juvenile hormone has been shown to exert a juvenilizing effect in the blowfly (Srivastava and Gilbert, 1969), the usual juvenile hormone effects are not found in *Drosophila*, even *in situ* (Bryant and Sang, 1968). It would be interesting to examine the effects of juvenile hormone in this system in view of the difficulty of demonstrating juvenile hormone effects in *Drosophila*.

The question arises whether the increase in size of ecdysterone-treated discs is due to cell division. Nearly all of the enlargement can be accounted for by the process of eversion. Poodry and Schneiderman (in preparation) have suggested that changes in cell size and shape, rather than changes in cell number, are primarily responsible for eversion *in situ*. Imaginal discs of *Drosophila* (Enzmann and Haskins, 1938), *Pieris brassicae* (Eassa, 1953), Saturniid silk moths (Krishnakumaran, Berry, Oberlander and Schneiderman, 1967; Patel and Madhavan, 1969) undergo increase in cell number, DNA synthesis, or increase in weight continuously, and independent of the molt cycle. In addition, ecdysterone does not cause DNA synthesis in *Galleria* wing discs *in vitro*, although ecdysone does (Oberlander, 1969). Thus, it is likely that in our experiments and perhaps *in situ*, imaginal discs do not respond to molting hormone by increased cell division except in the case of regeneration (Madhavan and Schneiderman, 1969).

Hadorn (1966) and his colleagues have performed extensive and elegant experiments on the stability of the determined state. The technique involves *in vivo* culture of *Drosophila* imaginal discs in the adult abdomen with periodic tests of the developmental potential of the cell by transplantation of the cultured imaginal disc fragments into larvae, where they metamorphose simultaneously with the host. This metamorphosis test has several limitations: (1) larvae tolerate only small needles and hence only relatively small imaginal disc fragments can be assayed. (2) To obtain a large amount of growth before metamorphosis, it is necessary to transplant the tissue into very young larvae, which are much more difficult to inject than adults. (3) Cutting a large fragment obtained after growth in an adult (in order to get pieces small enough to transplant into larvae) kills cells, distorts the pattern of bristles, and interposes an extra step in the experimental protocol. By employing analogues of ecdysone, the metamorphosis test can be simplified, by using adults instead of larvae, thus permitting metamorphosis of very large fragments. This promises to simplify investigation of certain problems of *Drosophila* development. The results also suggest that unless some other tissue is an intermediary for ecdysone action, *in vitro* metamorphosis of imaginal discs induced by ecdysterone should be possible, a problem currently under investigation.

Summary

The effects of molting hormone on the development of imaginal discs of *Drosophila melanogaster* were investigated. Imaginal leg discs from mature larvae were transplanted into the abdomens of fertilized adult female flies. The hosts were injected with a solution of a phytoecdysone—ecdysterone or cyasterone. The results showed: (1) Large doses of ecdysterone (over 1200 μg/g $= 1.2$ μg/fly) caused complete metamorphosis of the transplanted imaginal disc, including eversion

of the disc, formation of a pupal cuticle, and formation of adult cuticle with bristles, hairs and sensilla arranged in normal orientation.   (2) Ecdysterone was necessary as a sustained stimulus, and did not act merely as a trigger to metamorphosis. (3) Cyasterone was a more potent molting hormone than ecdysterone.   The technique promises to simplify investigation of pattern formation and other developmental problems in *Drosophila*.

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